Rapid desensitization of adrenaline- and neuropeptide Y-stimulated Ca^{2+} mobilization in HEL-cells

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1 Desensitization of G_i -coupled receptors, the β_2 -adrenoceptor for example, involves rapid and slower components but little is known regarding the existence of rapid desensitization of G_i -coupled receptors and its possible mechanisms. In HEL-cells stimulation of α_{2A} -adrenoceptors by adrenaline or Y_1 -like neuropeptide Y receptors by neuropeptide Y, transiently mobilizes Ca^{2+} from intracellular stores via a G_i -protein. We have used this model to study the existence and possible mechanisms of rapid desensitization of a G_i -mediated cellular response.

2 Following stimulation by adrenaline or neuropeptide Y Ca^{2+} levels returned towards baseline a few minutes after agonist addition and were refractory to a second agonist exposure demonstrating rapid desensitization. Cross-desensitization experiments with neuropeptide Y, adrenaline and moxonidine demonstrated the presence of homologous (both receptors) and heterologous desensitization (neuropeptide Y receptors only), and that the α_{2A} -adrenoceptor desensitization was not specific for phenylethylamine (adrenaline) or imidazoline agonists (moxonidine).

3 The protein kinase C activator, phorbol ester, rapidly desensitized the hormonal Ca^{2+} responses and inhibitors of protein kinase C enhanced the hormonal responses inconsistently. The tyrosine kinase inhibitor, herbimycin, enhanced Ca^{2+} mobilization by adrenaline and neuropeptide Y, whereas the protein phosphatase inhibitor, okadaic acid, did not affect Ca^{2+} mobilization or its desensitization.

4 In the absence of extracellular Ca^{2+} the endoplasmic reticulum Ca^{2+} -ATPase inhibitor, thapsigargin, reduced hormone-stimulated Ca^{2+} elevations, demonstrating that mobilization occurs from a thapsigargin-sensitive pool in the endoplasmic reticulum. The inositol phosphate-independent Ca^{2+} release modulator, ryanodine, significantly enhanced adrenaline- and neuropeptide Y-stimulated Ca^{2+} elevations. Blockade of the endoplasmic reticulum Ca^{2+} -ATPase by thapsigargin in the presence of extracellular Ca^{2+} enhanced hormone-stimulated Ca^{2+} increases, demonstrating the importance of this enzyme for the termination of the Ca^{2+} signal.

5 It is concluded that adrenaline and neuropeptide Y-stimulated Ca^{2+} mobilization in HEL-cells occurs from a thapsigargin- and ryanodine-sensitive store in the endoplasmic reticulum and desensitizes rapidly; this appears to involve multiple mechanisms including protein kinases, possibly acting on receptors, and Ca^{2+} release and sequestration mechanisms.

Keywords: α_2 -Adrenoceptor; neuropeptide Y receptor; Ca^{2+} mobilization; desensitization

Introduction

Desensitization is a general physiological mechanism by which cells adapt to a changing environment. Desensitization in response to agonist exposure has been best studied for a G_s -coupled receptor, the β_2 -adrenoceptor (Lefkowitz *et al.*, 1990; Lorenz *et al.*, 1991). It involves rapid and slower components which are at least partly mediated by the action of multiple protein kinases. Much less is known regarding the desensitization of G_i -coupled receptors. Most previous studies on the regulation of G_i -coupled receptor function have focused on slow desensitization, i.e. that occurring within hours or days (Green *et al.*, 1990; Wojcikiewicz & Nahorski, 1991; Shreve *et al.*, 1991). Whether rapid desensitization exists for G_i -coupled receptors and how it may occur is largely unknown. This may be due in part, to the fact that the classical signalling pathway of G_i -coupled receptors, inhibition of adenylate cyclase, is difficult to study at early time points.

We have recently demonstrated that G_i -coupled receptors such as the α_{2A} -adrenoceptor and the Y_1 -like neuropeptide Y (NPY) receptor in HEL-cells can, in addition to inhibition of adenylate cyclase, also couple to mobilization of Ca^{2+} from intracellular stores (Motulsky & Michel, 1988; Michel *et al.*, 1989). Similar Ca^{2+} elevations have been shown in SK-N-

MC neuroblastoma cells (Aakerlund *et al.*, 1990; Feth *et al.*, 1991), cultured neurones (Ewald *et al.*, 1988; Perney & Miller, 1989), pituitary cells (Shangold & Miller, 1990; Crowley *et al.*, 1990) and smooth muscle cells (Mihara *et al.*, 1989; Erdbrügger *et al.*, 1993). These Ca^{2+} elevations occur in less than 1 min, are mediated by a G_i -protein, and do not involve a Na^+/H^+ -antiporter or cyclo-oxygenase or lipoxygenase products of arachidonic acid (Motulsky & Michel, 1988; Michel *et al.*, 1989). Our previous data suggest that inositol phosphates are not involved (Motulsky & Michel, 1988; Michel *et al.*, 1989) but this is controversial (Daniels *et al.*, 1989). The α_{2A} -adrenoceptor and NPY-receptor-stimulated Ca^{2+} mobilization in HEL-cells and other cell types is only transient and usually lasts less than 3 min. Therefore, this may be a suitable model to investigate the existence of rapid desensitization of G_i -coupled receptor function and its possible mechanisms.

Methods

Cell culture

HEL-cells were originally obtained from Dr Papayannopoulou (Dept. Medicine, University of Washington, Seattle, WA, U.S.A.) and maintained in the chemically defined CG medium (Camon, Wiesbaden, Germany) in an atmosphere of

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95% air/5% CO₂ at 37°C at a density of $4\text{--}6 \times 10^5$ cells ml⁻¹ by daily dilution with fresh medium. In some experiments phorbol-myristate-acetate, herbimycin or dimethylsulphoxide were added to the culture medium in the indicated concentrations for the indicated times.

Ca²⁺ measurements

The free intracellular Ca²⁺ concentration was determined with the fluorescent inhibitor dye, fura-2, in a Hitachi F2000 spectrofluorometer as previously described (Feth *et al.*, 1992). Briefly, experiments were performed in buffer of the following composition (mM): HEPES 20, NaCl 120, KH₂PO₄ 5, Mg acetate 1, CaCl₂ 1 and glucose 1 mg ml⁻¹ at pH 7.4 using approximately $6\text{--}8 \times 10^5$ cells ml⁻¹. After 1 h of loading with the dye the cells were washed twice, resuspended in fresh buffer and used for fluorescence measurements within the next hour. Excitation was alternating at 340 and 380 nm with emission being read at 510 nm. Unless otherwise indicated, fluorescence data were converted into Ca²⁺ concentrations with software supplied by the manufacturer.

In some experiments a single-wavelength method with excitation at 340 nm only was used as previously described (Motulsky & Michel, 1988). In this case fluorescence data were converted into Ca²⁺ concentrations with the formula

$$[\text{Ca}^{2+}] = K \times (F - F_{\min}) / (F_{\max} - F)$$

In this formula, *K* is the affinity of fura-2 for Ca²⁺ ions, 224 nM, *F* is the fluorescence of the intracellular fura-2, *F*_{max} is its fluorescence when saturated with Ca²⁺, and *F*_{min} is its fluorescence in the absence of Ca²⁺.

Unless otherwise indicated compounds were added to the cell suspension 30–60 s before the receptor agonists. In the thapsigargin experiments receptor agonists were added when the thapsigargin effects on intracellular Ca²⁺ had reach a plateau, i.e. after 3–5 min. In some experiments, extracellular Ca²⁺ was chelated by addition of 5 mM EGTA 30–60 s before the other agents. Dimethylsulphoxide and herbimycin were added to the culture medium 4 days and 1 day respectively before the experiment. All drugs were added to the assay from stock solutions which were concentrated 100 fold in order to minimize dilution of the cell suspension. Whenever solvent concentrations reached (dimethylsulphoxide and methanol) or exceeded (other solvents) 0.1% in the final assay, samples were compared to vehicle-treated controls. All experiments were performed at room temperature to minimize dye leakage from the cells.

Chemicals

Fura-2 AM (obtained from Molecular Probes, Eugene, OR, U.S.A.) was dissolved at 1 mM in dimethylsulphoxide and used at 1 μM to load the cells. Adrenaline (obtained from Sigma, Munich, Germany) and NPY (obtained from Saxon Biochemicals, Hannover, Germany) were dissolved (at 10 and 1 mM, respectively) and further diluted in 10 mM HCl. Adrenaline was dissolved fresh daily whereas NPY solutions were stored at 4°C for up to three months; no decline in NPY activity could be observed during this period as assessed by its EC₅₀ to mobilize Ca²⁺ in HEL-cells. Thapsigargin (obtained from Calbiochem, La Jolla, CA, U.S.A.) was dissolved to 10 mM with dimethylsulphoxide, diluted to 1 mM with ethanol and then further diluted in 10 mM HCl. Herbimycin (from Gibco, Eggenstein, Germany) was dissolved at 1.75 mM in dimethylsulphoxide and diluted with cell culture medium. Ryanodine (obtained from Research Biochemicals Inc., Natick, MA, U.S.A.) was dissolved at 10 mM with methanol and diluted with buffer. The phorbol ester, phorbol-12-myristate-13-acetate (obtained from Calbiochem), was dissolved at 1 mM in dimethylsulphoxide and further diluted in water. H8 (N-[2-(methylamino)-ethyl]-5-isoquinolinesulphonamide dihydrochloride; obtained from Research Biochemicals Inc.) was dissolved at 10 mM in dilute

HCl. Staurosporine (obtained from Sigma) and calphostin C (obtained from Calbiochem) were dissolved at 10 mM in dimethylsulphoxide and further diluted in water.

Data evaluation

Data are given as mean ± s.e.mean of *n* experiments. The statistical significance of differences between control and treated cells determined in paired two-tailed *t* tests using the InStat programme (GraphPAD Software, San Diego, CA, U.S.A.).

Results

Nearly maximally effective concentrations of adrenaline and NPY (1 μM and 100 nM, respectively) increased intracellular free Ca²⁺ in HEL-cells with NPY responses being approximately 2.5 times those of adrenaline (192 ± 8 nM vs 73 ± 10 nM peak Ca²⁺ increase; *n* = 11; Figure 1). The agonist-stimulated Ca²⁺ increases were only transient; they peaked within 20–40 s and thereafter declined, reaching values close to baseline within 2–3 min (Figure 1). A second addition of a receptor agonist 3 min after the first exposure elicited responses of only $26 \pm 7\%$ (adrenaline) or $3 \pm 1\%$ (NPY) of the first (*n* = 11; Figure 1, Table 1). This desensitized state towards adrenaline and NPY was maintained for at least 30 min following washout of the agonists.

Since adrenaline and NPY appear to use similar mechanisms to increase Ca²⁺ in HEL-cells (Motulsky & Michel, 1988; Michel *et al.*, 1989), we determined whether desensitization of the response to one hormone also desensitizes the response to the other: 30 s after the second dose of adrenaline a dose of NPY was administered and in other cuvettes 30 s after the second dose of NPY a dose of adrenaline was given. Following two additions of adrenaline the subsequent response to NPY was not significantly altered (Figure 1, Table 1); following two additions of NPY the subsequent response to adrenaline, however, was considerably reduced (Figure 1, Table 1). Thus, adrenaline and NPY preferentially desensitize the responsiveness of their own receptors but the more efficacious Ca²⁺ mobilizer, NPY, can also partially (~80%) desensitize the adrenaline response.

The phenylethylamine agonist, adrenaline (1 μM), and the

Figure 1 Representative traces showing Ca²⁺ mobilization by adrenaline (Ad, 1 μM) and neuropeptide Y (NPY, 100 nM) in HEL cells. Also shown is the cross desensitization of the agonists.

imidazoline agonist, moxonidine (100 μ M), yielded quantitatively similar homologous desensitization of the α_{2A} -adrenoceptor-mediated response (Table 1). In cross-desensitization experiments adrenaline and moxonidine desensitized responses to each other to the same extent as their own responses (Table 1).

A 4-day treatment with 1.25% dimethylsulphoxide in the culture medium did not significantly alter basal intracellular Ca²⁺ levels (72 ± 4 nM in control vs. 64 ± 2 nM in treated cells, $n = 11$, $P > 0.05$) but attenuated adrenaline- and NPY-stimulated peak Ca²⁺ increases by 30% ($n = 5$, $P = 0.0226$) and 54% ($n = 6$, $P < 0.0001$; Table 2), respectively; the time course of the Ca²⁺ elevations, however, was not appreciably altered.

Addition of the protein kinase C activator, phorbol-12-myristate-13-acetate, 30 s before adrenaline or NPY, concentration-dependently inhibited Ca²⁺ mobilization; threshold inhibition occurred at 10 nM phorbol ester, and 100 nM inhibited the effects of adrenaline and NPY by $75 \pm 3\%$ and $60 \pm 5\%$, respectively (Figure 2, Table 2).

Among the three protein kinase C inhibitors tested (100 μ M H8, 1 μ M staurosporine, 1 μ M calphostin C) only staurosporine significantly enhanced the Ca²⁺ response to adrenaline and only staurosporine and calphostin C enhanced the response to NPY (Table 2); the duration of the Ca²⁺ increases was not consistently prolonged by any protein kinase C inhibitor and lower staurosporine concentrations

did not significantly alter peak receptor agonist-stimulated Ca²⁺ increases.

Cells were also treated with 100 nM phorbol-12-myristate-13-acetate overnight in order to down-regulate the cellular protein kinase C. Phorbol ester treatment desensitized stimulated Ca²⁺ elevations (13 ± 4 vs. 61 ± 7 nM for adrenaline and 14 ± 4 vs. 124 ± 17 nM for NPY in treated and control cells, respectively; $n = 3$ each, $P < 0.05$ vs. vehicle-treated cells).

In HEL-cells treated for 24 h with 1 μ M of the irreversible tyrosine kinase inhibitor, herbimycin (June *et al.*, 1990), both adrenaline and NPY caused greater peak Ca²⁺ increases than in vehicle-treated cells (Table 2).

Okadaic acid (1 μ M), a protein phosphatase inhibitor (Mattingly & Garrison, 1992), by itself induced a minor elevation of intracellular Ca²⁺ (15 ± 1 nM, $n = 8$) but this was not different from that induced by vehicle (1% ethanol, 14 ± 1 nM, $n = 8$). The Ca²⁺ elevations in response to adrenaline and NPY, however, were not affected by addition of okadaic acid 1 min before the hormones (Table 2).

Addition of 30 mM KCl did not affect the basal intracellular Ca²⁺ concentration but reduced adrenaline- and NPY-stimulated Ca²⁺ increases by $68 \pm 3\%$ and $30 \pm 4\%$, respec-

Table 1 Cross-desensitization of Ca²⁺ mobilization by neuropeptide Y (NPY), adrenaline and moxonidine in HEL cells

First agonist	Second agonist	% desensitization
NPY	NPY	97 ± 1
NPY	adrenaline	82 ± 4
Adrenaline	NPY	2 ± 6
Adrenaline	adrenaline	74 ± 7
Adrenaline	moxonidine	81 ± 9
Moxonidine	adrenaline	82 ± 3
Moxonidine	moxonidine	78 ± 5

The ability of a first agonist (1 μ M adrenaline, 100 μ M moxonidine or 100 nM NPY) to desensitize responses to a second agonist was tested. When intracellular Ca²⁺ had returned to baseline (usually within 3–5 min) after addition of an agonist, a second agonist was added. Desensitization of the response to the second agonist was determined as % reduction in its response when compared to its response in control cells. Data are mean $\pm$ s.e.mean of 7–11 experiments. A representative tracing of such experiments is shown in Figure 1.

Figure 2 Effect of phorbol-12-myristate-13-acetate (PMA) added 30 s before adrenaline (1 μ M, $\circ$) or neuropeptide Y (NPY, 100 nM, $\blacksquare$) on the agonist-stimulated peak Ca²⁺ increases, expressed as % of those obtained in matched cuvettes in the absence of PMA. Control Ca²⁺ increases were 40 ± 6 and 156 ± 9 nM for adrenaline and NPY, respectively. Data are the mean $\pm$ s.e.mean of 4–5 experiments.

Table 2 Effects of various compounds on adrenaline- and neuropeptide Y (NPY)-stimulated Ca²⁺ mobilization in HEL-cells

	Adrenaline (1 μ M)	NPY (100 nM)
	response in % of control	
KCl (30 mM)	$32 \pm 3^*$ (3)	$70 \pm 4^*$ (4)
NaCl (30 mM)	$32 \pm 3^*$ (3)	$67 \pm 5^*$ (5)
Thapsigargin (50 nM)	$155 \pm 18^*$ (7)	$288 \pm 68^*$ (10)
Ryanodine (10 μ M)	$142 \pm 10^*$ (12)	$120 \pm 6^*$ (11)
DMSO treatment (1.25%, 4 days)	$70 \pm 4^*$ (5)	$46 \pm 4^*$ (6)
Phorbol ester (100 nM)	$24 \pm 3^*$ (5)	$40 \pm 5^*$ (4)
H8 (100 μ M)	106 ± 22 (5)	84 ± 10 (6)
Staurosporine (1 μ M)	$142 \pm 15^*$ (6)	$122 \pm 8^*$ (6)
Calphostin C (1 μ M)	110 ± 9 (5)	$124 \pm 7^*$ (6)
Herbimycin (1 μ M, 24 h)	$188 \pm 35^*$ (7)	$138 \pm 5^*$ (8)
Okadaic acid (1 μ M)	104 ± 13 (4)	99 ± 3 (4)

All compounds were added 30–60 s before adrenaline or NPY except for dimethylsulphoxide (DMSO) and herbimycin which were added to the culture medium 4 days and 1 day beforehand, respectively, and washed out prior to the Ca²⁺ measurements. Phorbol-12-myristate-13-acetate was the phorbol ester. Data are mean $\pm$ s.e.mean of the number of experiments indicated in parentheses. Data are expressed as % of the Ca²⁺ increase observed in paired vehicle-controlled cells. * $P < 0.05$.

Table 3 Ca^{2+} elevations caused by thapsigargin, adrenaline, neuropeptide Y (NPY) and their combinations in the presence and absence of extracellular Ca^{2+}

	$[\text{Ca}^{2+}]$ 1 mM	$[\text{Ca}^{2+}]$ 0 mM (5 mM EGTA added)
Thapsigargin (50 nM)	564 ± 46	425 ± 32
Adrenaline (1 μM)	56 ± 10	119 ± 17
Adrenaline following thapsigargin	$140 \pm 25^*$	$25 \pm 33^*$
NPY (100 nM)	145 ± 25	353 ± 79
NPY following thapsigargin	$323 \pm 55^*$	$154 \pm 43^*$

Ca^{2+} increases were determined by a single-wave-length method in standard buffer containing 1 mM Ca^{2+} or following chelation of extracellular Ca^{2+} by addition of 5 mM EGTA 30 s before the addition of thapsigargin adrenaline or NPY. Data are mean $\pm$ s.e.mean of 7 experiments (15–17 for effects of thapsigargin alone), and Ca^{2+} increases are expressed in nM. * $P < 0.05$ vs results obtained in the absence of thapsigargin.

Figure 3 Representative effects of 50 nM thapsigargin on neuropeptide Y (NPY)-stimulated Ca^{2+} mobilization in HEL cells. NPY-stimulated Ca^{2+} elevations were 186 ± 11 nM in the absence, and 287 ± 26 nM in the presence, of thapsigargin ($n = 7$; $P < 0.05$).

tively. Similar data were obtained with addition of 30 mM NaCl (Table 2).

In standard buffer containing 1 mM Ca^{2+} and using the double-wave-length method, 50 nM thapsigargin increased intracellular Ca^{2+} from 77 ± 2 to 405 ± 39 nM (mean increase 328 ± 38 nM, $n = 19$). Despite the already elevated cytoplasmic Ca^{2+} concentration, adrenaline and NPY further increased intracellular Ca^{2+} and these increases significantly exceeded those in the absence of thapsigargin (Figure 3, Table 2). Similar data were obtained in the presence of extracellular Ca^{2+} by the single-wave-length method (Table 3). In Ca^{2+} -free buffer, 50 nM thapsigargin elicited slightly smaller Ca^{2+} elevations than were seen in Ca^{2+} -containing buffer (Table 3) as determined by the single-wave-length method. The subsequent Ca^{2+} responses to adrenaline and NPY in Ca^{2+} -free buffer were significantly reduced in the presence of thapsigargin (Table 3).

Ryanodine, an inositol phosphate-independent modulator of Ca^{2+} release, did not alter the basal intracellular Ca^{2+} concentration. Ryanodine enhanced adrenaline-stimulated peak Ca^{2+} increases from 65 ± 6 to 89 ± 8 nM ($n = 12$, $P = 0.0008$, Table 2) and NPY-stimulated Ca^{2+} increases from 215 ± 18 to 253 ± 17 nM ($n = 11$, $P < 0.0060$, Table 2).

Discussion

The mechanisms involved in the desensitization of G_i -coupled receptors, for example the β_2 -adrenoceptor, have been extensively characterized (Lefkowitz *et al.*, 1990; Lorenz *et al.*, 1991). It is now clear that β_2 -adrenoceptor desensitization involves rapid (uncoupling and sequestration for example) and slower components (down-regulation for example) which are mediated at least partly by protein kinases such as adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent protein kinase or β -adrenoceptor kinase, which specifically recognizes the agonist-occupied form of the receptor. In contrast, little is known of the regulation of G_i -coupled receptors. Previous studies have demonstrated that prolonged agonist exposure may down-regulate some G_i -coupled receptors such as A_1 adenosine (Green *et al.*, 1990) and muscarinic receptors (Wojcikiewicz & Nahorski, 1991) or α_2 -adrenoceptors (Shreve *et al.*, 1991). Whether rapid desensitization of G_i -coupled receptor function also exists is unclear, and for NPY receptor function neither rapid nor slow desensitization has been studied.

Since an inhibitory response, inhibition of adenylate cyclase, is difficult to study at early time points, we have used a stimulatory response, elevation of intracellular Ca^{2+} , to investigate the existence and possible underlying mechanisms of rapid desensitization of G_i -coupled receptors. In HEL-cells, adrenaline and NPY increase intracellular Ca^{2+} via α_{2A} -adrenoceptors and Y_1 -like receptors, respectively, and both responses are G_i -coupled (Motulsky & Michel, 1988; Michel *et al.*, 1989). Adrenaline- and NPY-stimulated Ca^{2+} elevations are only transient and return towards baseline within a few minutes. Our data show that the rapid decline of intracellular Ca^{2+} concentration in the continued presence of agonist is caused by desensitization rather than by removal or inactivation of the agonist since the HEL-cells remained unresponsive to a second dose of agonist. Thus, rapid desensitization exists for α_2 -adrenergic and NPY receptor-mediated Ca^{2+} elevations in HEL-cells.

Desensitization of receptor responsiveness can occur at multiple levels including receptors, G-proteins and effector enzymes. Our cross-desensitization experiments with adrenaline and NPY demonstrate that adrenaline induced only homologous desensitization whereas NPY also caused considerable heterologous desensitization of the adrenaline response. This may be related to the fact that NPY is a considerably more effective agonist which may in turn be related to the fact that HEL-cells have only approximately 6,000 α_2 -adrenoceptors (McKernan *et al.*, 1987) but more than 40,000 NPY receptors per cell (Feth *et al.*, 1992). Homologous desensitization of the α_2 -adrenoceptor response is not specific for phenylethylamine or imidazoline agonists since adrenaline and moxonidine desensitized each others responses to the same extent as their own. Thus, the previously reported imidazoline-specific mechanisms of α_2 -adrenoceptor desensitization in vascular smooth muscle

(Ruffolo *et al.*, 1977) are not involved in the desensitization mechanism in HEL-cells.

Differentiation with dimethylsulphoxide activates protein kinase C in murine erythroleukaemia cells (Chakraverthy *et al.*, 1992) and accelerates the desensitization rate of G_i -coupled prostaglandin E receptors in human erythroleukaemia cells (HEL-cells; Ashby *et al.*, 1991). This differentiation also lowered peak adrenaline- or NPY-stimulated Ca^{2+} elevations, which is compatible with the idea of enhanced desensitization rates for these G_i -mediated responses. HEL-cells contain the β - and δ -forms of protein kinase C, and activation of the enzymes can desensitize Ca^{2+} mobilization by other receptors in these cells (Grabarek *et al.*, 1992; Brass, 1992). Our data show that phorbol ester rapidly desensitizes adrenaline- and NPY-stimulated Ca^{2+} elevations. Desensitization of NPY-stimulated Ca^{2+} elevations by phorbol ester has also been demonstrated by other investigators, who additionally found that it can be prevented by specific protein kinase C inhibitors (Daniels *et al.*, 1992) indicating that it is mediated by protein kinase C. The structural basis for this desensitization may be the presence of protein kinase C phosphorylation sites in the cytoplasmic domains of α_{2A} -adrenoceptors (Kobilka *et al.*, 1987) and Y_1 -like NPY receptors (Krause *et al.*, 1992). It is also possible that phorbol ester exposure stimulates Ca^{2+} uptake into the endoplasmic reticulum, since it has recently been shown that phorbol ester enhances and that the protein kinase C inhibitor, calphostin C, reduces the V_{max} of this Ca^{2+} removal mechanism in human platelets (Tao *et al.*, 1992).

The desensitization of adrenaline- and NPY-stimulated Ca^{2+} elevations by phorbol ester, however, does not necessarily imply that protein kinase C activation is important in the rapid desensitization of agonist-stimulated Ca^{2+} mobilization. The protein kinase C inhibitors staurosporine, H8 and calphostin C yielded contradictory results and none of them completely prevented desensitization. The protein kinase C inhibitor, H7, also failed to prevent desensitization of NPY-stimulated Ca^{2+} mobilization in HEL-cells, although it effectively prevents the desensitization caused by phorbol ester (Daniels *et al.*, 1992). Together these data indicate that activation of protein kinase C is unlikely to play a major role in the rapid desensitization of adrenaline- or NPY-stimulated Ca^{2+} mobilization.

The human Y_1 -like NPY receptor contains a consensus site for tyrosine phosphorylation in its cytoplasmic domain (Larhammar *et al.*, 1992). Our data demonstrate that the specific tyrosine kinase inhibitor, herbimycin, enhanced adrenaline- and NPY-stimulated Ca^{2+} mobilization, and thus are consistent with the idea that a tyrosine kinase may participate in the rapid desensitization of Ca^{2+} mobilization. However, these data are not easy to interpret on a molecular level since a 24 h incubation with herbimycin was necessary to achieve this effect, and because the substrate of tyrosine kinase activity may also be G-protein α -subunits which can undergo tyrosine phosphorylation (Hausdorff *et al.*, 1992). The overall role of phosphorylation events in the rapid

desensitization of adrenaline- or NPY-stimulated Ca^{2+} mobilization remains unclear since the protein phosphatase inhibitor, okadaic acid, had no detectable effects, although similar okadaic acid treatment enhances desensitization of thrombin-stimulated Ca^{2+} elevations in HEL-cells (Brass, 1992).

The simultaneous presence of homologous and heterologous desensitization suggests that alterations downstream of the receptors may also be involved. Candidates for this would be the Ca^{2+} release and sequestration mechanisms. In some cells depolarization impairs the responses to Ca^{2+} elevating agonists (Randriamampita *et al.*, 1991). In HEL-cells, depolarization by addition of a high potassium concentration impaired Ca^{2+} responses to adrenaline and NPY, but addition of an equal concentration of sodium had the same effect. Thus, osmolarity rather than depolarization or membrane potential appears to be involved in this attenuation.

Intracellular Ca^{2+} stores can be depleted when the inhibitor of the endoplasmic reticulum Ca^{2+} -ATPase, thapsigargin, is applied to cells in Ca^{2+} -free buffer (Thastrup *et al.*, 1990) and treatment with this agent attenuated adrenaline- and NPY-stimulated Ca^{2+} mobilization. Qualitatively similar data for NPY-stimulated Ca^{2+} increases in HEL- and SK-N-MC-cells have been described (Aakerlund *et al.*, 1990; Daniels *et al.*, 1992). In the endoplasmic reticulum thapsigargin acts on both inositol phosphate-sensitive and -insensitive compartments (Thastrup *et al.*, 1990; Lytton *et al.*, 1991) and adrenaline and NPY mediate Ca^{2+} mobilization independently of inositol phosphates (Motulsky & Michel, 1988; Michel *et al.*, 1989). A modulator of inositol phosphate-insensitive Ca^{2+} release, ryanodine (Sorrentino & Vople, 1993), enhanced adrenaline- and NPY-stimulated Ca^{2+} mobilization. Together these data suggest that depletion of intracellular Ca^{2+} pools may desensitize adrenaline- and NPY-stimulated Ca^{2+} mobilization. However, we consider it unlikely that this plays a role in the rapid desensitization following agonist addition because it is seen in Ca^{2+} -free and in Ca^{2+} -containing buffer, whereas depletion of the store apparently occurs only in Ca^{2+} -free buffer.

Our results demonstrate that adrenaline- and NPY-stimulated Ca^{2+} elevations rapidly desensitize in HEL-cells. This involves homologous and heterologous components. Ca^{2+} increases by both agonists are enhanced by the endoplasmic reticulum Ca^{2+} -ATPase inhibitor, thapsigargin, in the presence of extracellular Ca^{2+} , by the inositol phosphate-independent Ca^{2+} release modulator, ryanodine, and by the tyrosine kinase inhibitor, herbimycin. They can be attenuated by increased osmolarity, Ca^{2+} store depletion, and a protein kinase C activating phorbol ester. Whether protein kinase C activation plays an important role in the rapid desensitization following agonist exposure remains unclear since protein kinase C inhibitors had inconsistent effects.

This work was supported by the Deutsche Forschungsgemeinschaft.

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(Received June 21, 1993)

Revised December 6, 1993

Accepted February 16, 1994)